THE FERTILIZATION OF AMŒBA PROTEUS.

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Three years ago I published a short paper entitled "Evidences of a Sexual Cycle in the Life-history of *Amœba proteus*"[1] in which I described the formation of chromidium in one life-phase of this common rhizopod and the subsequent formation of secondary nuclei. The latter were interpreted as nuclei of the supposed gametes which were collected in a cyst. The supposed gametes were in no case seen to emerge from the cyst and conjugation was not observed. Many of the structures observed in the amœbæ at this time, could not be interpreted in terms of the corresponding phases of other fresh water rhizopods, a curious division of the granules (represented in Plate 3, Fig. 23 of the former paper), and an equally enigmatical series of spheres with peripheral granules (represented in Figs. 12, 24 and 27), being particularly hard to homologize with other known phases in rhizopod development. Had I not been busy with other work at the time, I might have discovered that the very material used for these "evidences" would furnish proof of the actual fertilization, for this last spring, hoping to find some trace of a maturation process in rhizopods at the period of chromidium formation, I dissolved off the cover glasses from the amœbæ which were preserved in balsam, embedded them in paraffine, cut them one by one in sections from three to five microns in thickness and discovered the method of fertilization. A careful examination of the so-called "dividing granules" in these sections revealed the fact that what I had interpreted in the total mounts as dividing forms of the chromidial granules, were actually minute nuclei in the process of fusion, and that, instead of division, it was the process of fertilization, while the encysted bodies with the peripheral granules were stages in the development of these fertilized nuclei. The material, unfortunately, is still not complete enough to give the details of the chromatin changes as thoroughly as I wish, but there are enough stages to enable us to clear up this sexual phase

[1] *Arch. f. Protistenk.*, V., 1904, pp. 1–16.

in the cycle of *Amœba proteus* and to bring together the observation of Scheel and earlier observers and to combine them in one completed life history.

As described in my earlier paper, this phase of the life-history of *Amœba proteus* is characterized by the repeated division of the nucleus until many nuclei are present in the cell, 72 to 80 being the largest numbers observed in any one organism. These nuclei then fragment, and the chromatin granules, liberated by rupture of the nuclear membranes, are distributed in the cytoplasm. This fragmentation continues until all but one of these primary nuclei are thus broken up, this one remaining as a residual nucleus, while the cytoplasm becomes packed with the chromatin fragments which I had interpreted as the chromidium. Stages in this disintegration of the nuclei are shown in Figs. 6, 7, 8, 19–26, of my earlier paper. These granules were described as increasing in size, dividing and ultimately forming the hollow spheres with peripheral granules in the encysted stage (Figs. 23, 24 and 27 of the former paper).

Now that the amœbæ have been removed from the slides, sectioned, and stained in iron-hæmatoxylin, the structure of the granules is brought out with more vivid clearness than in the total preparations stained with picro-carmine. The disintegration of the primary nucleus can be followed step by step in the sections and the origin of the gametic nuclei, as I may now call them, can be easily traced.

The first indication of fragmentation is the collection of the chromatin about the inner walls of the primary nucleus. Comparatively large reservoirs are massed about the periphery in this way (Fig. 1), but in the meantime chromatin granules in the interior of the nuclei are assuming a definite form, while a less deeply staining, more homogeneous cortical zone of nuclear plasm collects around them. The peripheral granules are also used to form similar minute nuclei which, apparently as soon as formed, move out into the cytoplasm. Here they are clearly marked nuclei, consisting of a densely staining central granule or karyosome with a more faintly staining cortical zone (Figs. 2, 3, 4, 5, 6, 7). The bulk of the primary nuclei is metamorphosed into these secondary nuclei, which are so small and so numerous that they give a characteristic granular appearance to the cell.

The earliest stages of secondary nucleus formation within the nucleus are so minute that they would scarcely be taken for the same things as the cytoplasmic nuclei. Stages in growth, however, can be found in which the size varies from these extremely minute ones to the full size nuclei of the cytoplasm (Figs. 1, 2). Fig. 5 shows a primary nucleus in the process of fragmentation with two full size secondary nuclei emerging at *a* while within the nucleus one or more large ones can be made out. Figs. 6 and 7 and Fig. 8 show similar late stages in secondary nucleus formation.

After emerging from the primary nucleus the secondary or gametic nuclei fuse and the stages in the process can be followed step by step in the fixed material. In such fixed material, however, the argument may be raised that it is equally possible to trace the history of stages in the opposite direction and claim that the process is one of division and not conjugation, this being one of the serious difficulties in working on preserved organisms. Nevertheless the evidence is so strong that there exists no doubt whatsoever in my own mind that we are dealing with conjugation and not with division. In the first place the number of gametic nuclei is far greater than the number of sporoblasts which make up the later cyst stage. In one specimen I counted more than three hundred gametic nuclei in addition to eighteen as yet unfragmented primary nuclei, while the number of sporoblasts in the later stages does not exceed 250 in any one of the specimens in my possession. Numerical relations indicate, therefore, that union rather than division takes place. In the second place the individuals of the pairs of nuclei that are fusing, are of the same size as the single ones. If they were dividing the daughter nuclei would be considerably smaller. Size here, however, is so variable that I do not lay much stress on this argument. In the third place, if the nuclei were dividing we should find dumb-bell shaped figures with the diameter of the nuclei drawn out at right angles to the plane of division. This is not the case, the minute nuclei remaining as spherical as though not in contact. In the fourth place we should expect to find connecting strands of chromatin substance between the recently divided karyosomes if it were a case of division, but no such connecting strands exist. In

the fifth place we should expect to find the daughter karyosomes elongated in the axis at right angles to the plane of division if it were division. Such is not the case as inspection of the figures shows, while in many cases the two karyosomes are elongated in an opposite direction (Fig 2, *d*; Fig. 3, *c*). In the sixth place if it were division we should expect it to take place more rapidly than the figures indicate, for in fusion, the process in protozoa requires a longer time than does division and the large number of double forms of these secondary nuclei indicates that the process is a relatively slow one.

On the whole, therefore, I believe the evidence justifies no other conclusion than that this is a process of fusion and not of division and that we are dealing here with an actual conjugation of nuclei. The fusion takes place by preliminary union of the extreme peripheries, this is followed by union of the homogeneous portions, and finally by union of the karyosomes (Figs. 2, 3). In one or two cases I have seen some evidence that more than two nuclei may thus fuse. Fig. 2, *e*, for example presents such a case, the larger size, and the two karyosomes indicating that fusion of two nuclei has already taken place. It occurred to me that the minute nuclei, before fusing, might possibly divide in some form of maturation division, but I have been unable to confirm this supposition in the material at hand, and incline to the belief that fusion occurs at once, for the uniting nuclei are abundant in the immediate vicinity of disintegrating primary nuclei.

The result of this fusion of gametic nuclei is, in each case, a nucleus of somewhat larger size in which the central granule fragments into a cloud of extremely minute chromatin granules lying about a central space which is the beginning of the vacuole characteristic of the later phases in development of the spores (Figs. 9, 10, 14). These granules next collect in small aggregates which are arranged about the periphery of the vacuolated mass, from 70 to 100 of them, as nearly as I can estimate, being formed in each of the many centers which now correspond to those multiplication centers of sporozoa called sporoblasts by Schaudinn. In this period, the sporulating centers or sporoblasts, are carried about in the cytoplasmic flow and appear as small

hollow nuclei which, in many cases, resemble the nuclei of some *Pelomyxa*-like forms. In the specimen from which Fig. 11 of my original paper was taken, there are more than 200 of these centers of multiplication, while in the encysted form shown in my original Fig. 12, a section of which is shown here in Figs. 13 and 14, there are about 250 of these sporoblasts.

The ultimate form assumed by the *Amœba* in the material which I possess, may be described as a collection of these sporoblasts, each one of which is a hollow sphere, the walls being studded with minute granular nuclei from 70 to 100 in number (Fig. 14). They lie about the one remaining primary nucleus which is shown in the section reproduced in Fig. 13.

A first examination of these nuclei in the sections gives the impression that the amœba body is well infested by parasites and this indeed, was my belief until a critical examination of the material in all stages. convinced me of my error. While under the belief that these nuclei were parasites I sought to interpret the several phases of the *Amœba* cycle which was described in 1904, as effects of such an infection. I concluded that minute parasites enter the body of *Amœba*, stimulate the nucleus to divide as does *Plasmodiophora brassicæ* the cell nuclei in the cabbage root, and then multiply by division in the interior of the endoplasm, the rapid multiplication filling the body with the minute granules which, earlier, I had interpreted as chromidium granules. My impression was strengthened by the observations of Schubotz[1] who interpreted the nuclei which I had described in *A. proteus*, as degenerating nuclei, an interpretation with which I quite agree, although not in the sense he meant. Prandtl[2] still more recently has published an interesting account of the development of young forms of *Allogromia* as parasites in the endoplasm of *Amœba proteus* and he also agrees with Schubotz that the nuclei described in my earlier paper are degenerating nuclei, and suggests that my "chromidium granules" may be young phases of an organism similar to the *Allogromia* which he describes.

While frankly admitting the possibility that these small nuclei may be parasites, a possibility which with fixed material and on

[1] "Beiträge zur Kenntnis der *Amœba blattæ* und *A. proteus*," *Arch. f. Prot.*, VI., 1905.

[2] "Der Entwicklungskreis von *Allogromia* sp.," *Arch. f. Prot.*, IX., 1907.

morphological grounds alone, cannot be entirely refuted, I firmly believe that they are not parasites but developmental phases of *Amœba proteus*. My reasons for this belief may be briefly summarized as follows: First, the early stages of nuclear increase are present in specimens of *Amœba* in which there are none of the supposed parasites. Second, the supposed parasites must originate inside of the supposed degenerating nuclei, for, as Figs. 2, 5, 6, 7 and 8 clearly show, the structures under consideration first arise inside of the nuclei, and wander outside by dissolution of the nuclear membrane. Third, if they are parasites they must conjugate inside of the endoplasm of the *Amœba*, for, as we have seen above, there are no good grounds for interpreting these structures as dividing forms. Fourth if the structures in question are conjugating parasites the conjugation leads to further development within the protoplasm of the host cell without any protecting membranes against the resistance of the host cell. Fifth, if these things are parasites, then the secondary nuclei of *Arcella*, *Polystomella* and *Entamœba* must likewise be parasites, for the resemblance between the several cases is too strong to allow another interpretation. Finally, if they are parasites, they must wander into the nuclei of *Amœba proteus* in the form of germs too small to be recognized and grow there into larger nucleus-like bodies which emerge from the nucleus and conjugate, and all this without disturbing the physiological equilibrium of the *Amœba*, and without effecting any pathological change such as formation of vacuoles or spaces about themselves (cf. Figs. 2, 3, 4). The evidence, therefore, is altogether in favor of the nuclear character of these questionable structures, and their union, I believe, can be interpreted in no other way than as the fertilization of this universal rhizopod.

The fertilization of *Amœba proteus* has been sought for by biologists for decades. Many observations have been published on phenomena supposed to be conjugation processes, but these in the main, have turned out to be cases of plastogamy, common amongst the rhizopods, or cases of engulfing of one by another. If the fertilization were any ordinary process similar to what occurs in other allied forms, there seems little likelihood that it would have been overlooked for these many years. But occurring

as I have now shown it to occur, in a manner quite unlike that of the majority of other rhizopods that we know, the reason for its being overlooked becomes apparent. Many have observed *Amœba proteus* in the multinucleate condition. Carter in 1863 observed as many as 70 nuclei in specimens of *Amœba princeps* which is usually regarded as the same as our *Amœba proteus*, and Wallich in the same year observed the liberation of many fine granular bodies by the rupture of the nuclear membrane, while Schaudinn [1] calls attention to the fact that he has observed the nuclear multiplication and suggests that it betokens a possible sexual phase.[2]

The process of fertilization, as I have described it here, comes under the head of the conjugation phenomena known as endogamy, or conjugation of nuclei within the original cell parent. It is not the only instance of such a phenomenon amongst the *Sarcodina*. Hertwig, in 1898, described self-fertilization in the case of *Actinosphærium*, while Schaudinn in 1903 (*loc. cit.*) described it in the case of *Entamœba coli*, the harmless commensal of the intestine. In both cases, however, the process is described as much more complicated than that which I have outlined here. In *Actinosphærium* the vegetative nuclei are reduced by fusion or by absorption to a relatively small number. The cell then divides into as many daughter cells as there are nuclei (five to ten); these daughter cells encyst within the parent cyst, the nucleus divides by mitosis and each of the cysts divides into two daughter cysts, each with one nucleus. The nucleus in each of these daughter cysts next divides twice, giving rise to two "polar body" equivalents. The cytoplasm and remaining nuclei of the two daughter cysts then fuse and the fertilization is completed by the reunion of the parts. In *Entamœba* the process is more like that of *Amœba* as described here. The cell throws out foreign matter and waste products of its own metabolism, and becomes smaller, more compact and more spherical. After secreting a gelatinous membrane in which the cell remains encysted, the cell nucleus divides into two nuclei, the division being followed by an incom-

[1] "Untersuchungen über die Fortpflanzung einiger Rhizopoden," *Arb. a. d. Kais. Gesundh.*, XIX., 1903.

[2] For further historical data see my original paper.

plete division of the cell body. After this division, the daughter nuclei fragment, forming a mass of minute chromidial granules which are distributed throughout the cell. From the two masses of granules thus formed secondary nuclei arise by fusion as in the case of *Centropyxis*, *Arcella*, *Polystomella*, and rhizopods generally, and these two nuclei, after preliminary division giving rise to what Schaudinn interprets as "polar body" equivalents, divide for a third time, the products of this division fusing two by two. The partly separated protoplasmic body then reunites and the fertilization cell contains two fertilization nuclei. These two nuclei then divide twice forming eight nuclei altogether and these finally become the nuclei of eight amœboid spores.

Except for the maturation divisions, which future study may reveal, the process of fertilization in *Amœba proteus* is thus strikingly similar to that of *Entamœba coli* the gametic nuclei arising by fragmentation instead of by division as in the latter case. The fertilization nucleus forms, not eight, but many daughter nuclei, and from analogy, I would expect these vacuolated centers of multiplication or sporoblasts in *Amœba proteus* to produce from 70 to 100 pseudopodiospores.

The formation of the secondary nuclei in *Amœba proteus* differs in some important respects from the process in other rhizopods. In *Arcella*, *Centropyxis* and *Polystomella* for example, it occurs in the chromatin substance that is either transfused through the membrane of the nucleus or formed by fragmentation of the nuclei. In all cases that have been worked out, however, the secondary nuclei are formed from the substance of this chromidium and we can thus trace their history back indirectly to the primary nuclei. In *Amœba proteus* on the other hand, the nuclei are not formed from diffused chromatin nor from the fragments of primary nuclei but they form directly within the primary nuclei and emerge from it as fully formed secondary nuclei. In this case, as in the other cases, the secondary nuclei are the gametic nuclei, only here their union is, so to speak, precocious and without the customary gamete formation, while the union and especially the further development *within the parent cell*, are unusual and unexpected discoveries.

Scheel takes up the life history of *Amœba proteus* from this

stage on. In his paper on the Encystment of *Amœba proteus*[1] he describes compound cysts which I would interpret as a late stage (with protecting gelatinous membranes), of the stage shown in my Fig. 12 of the original paper (section shown in Figs. 13, 14 of present paper). Each one of his cysts I would interpret as one of the sporoblasts (Fig. 14), which, by independent growth, reaches the size he describes (about 80 microns). The peripheral granules (Fig. 14, *b*) of my sporoblasts become his nuclei of the cyst and later, the nuclei of the minute amœbæ with stellate pseudopodia (*Amœba radiosa*).

In conclusion I would substitute for the tentative life-cycle published three years ago, the following: The ordinary *Amœba proteus* reproduces asexually by division (seen in every laboratory); ultimately the asexual cycle is replaced by the sexual, the conditions of which, periods, etc., are entirely unknown; the sexual cycle is inaugurated by the multiplication through division of the nuclei until many "primary" nuclei are formed; these primary nuclei fragment directly into minute granular nuclei corresponding to the "secondary" nuclei of *Polystomella*, *Centropyxis*, etc. In *Amœba* the secondary nuclei may be called the "gametic" nuclei; the gametic nuclei fuse to form fertilization nuclei; in these the fused karyosomes fragment to form finely divided chromatin (it is, strictly speaking, not a chromidium for it is entirely intra-nuclear), while a vacuole forms in the interior; this vacuolated fertilization nucleus becomes a center of multiplication (equivalent in every way to a sporozoön sporoblast); by accumulation of these fine chromatin granules the peripheral or "tertiary" nuclei are formed; the tertiary nuclei, surrounded by a minute bit of plasm, grow into the pseudopodiospores observed by Scheel (hypothetical); these young pseudopodiospores break away from the parent cyst and develop into young amœbæ formerly known as *Amœba radiosa*, and these, in turn, develop into the ordinary *Amœba proteus* of pond and laboratory.

COLUMBIA UNIVERSITY,
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[1] Festschrift für Carl von Kupffer, Jena, 1899.

Explanation of Plate XI.

The photographs are from sections, stained with iron-hæmatoxylin, of the same specimens of *Amœba proteus* that were pictured in my previous paper on "Evidences of a Sexual-cycle in the Life-history of *Amœba proteus*. The magnifications vary from 550 diameters (Fig. 13) to 2,000 diameters (Figs. 9 and 10).[1]

Fig. 1. Part of section of *Amœba* with about 30 primary nuclei some of which have begun to fragment. The chromatin is massed in a characteristic manner about the periphery while the small points in the center are the karyosomes of the future secondary nuclei. At this stage there are few secondary nuclei in the cytoplasm. × 1,000.

Fig. 2. Section from the amœba represented in Fig. 7 of my previous paper. The majority of the primary nuclei have fragmented and the cell body is spotted with the secondary nuclei. These may be seen *forming* in the large primary nucleus (*f*). The photograph also shows many stages in the fusion of the secondary nuclei. At (*a*) two are seen with the peripheries in contact; at (*b*) the bodies are beginning to fuse; at (*c*) and (*d*) fusion of bodies is completed, while the karyosomes have not yet fused but lie facing one another (if this were division the karyosomes would be elongated in the direction at right angles to this); at (*e*) there is an apparent double fertilization; at (*g*) the karyosome from the upper nucleus has migrated into the lower nucleus before fusion of the nuclei is complete. × 1,500.

Fig. 3. Primary nucleus with brood of young secondary nuclei within it and several secondary nuclei in various stages of fusion (*a*), (*b*) and (*c*) representing different stages in the process. × 1,350.

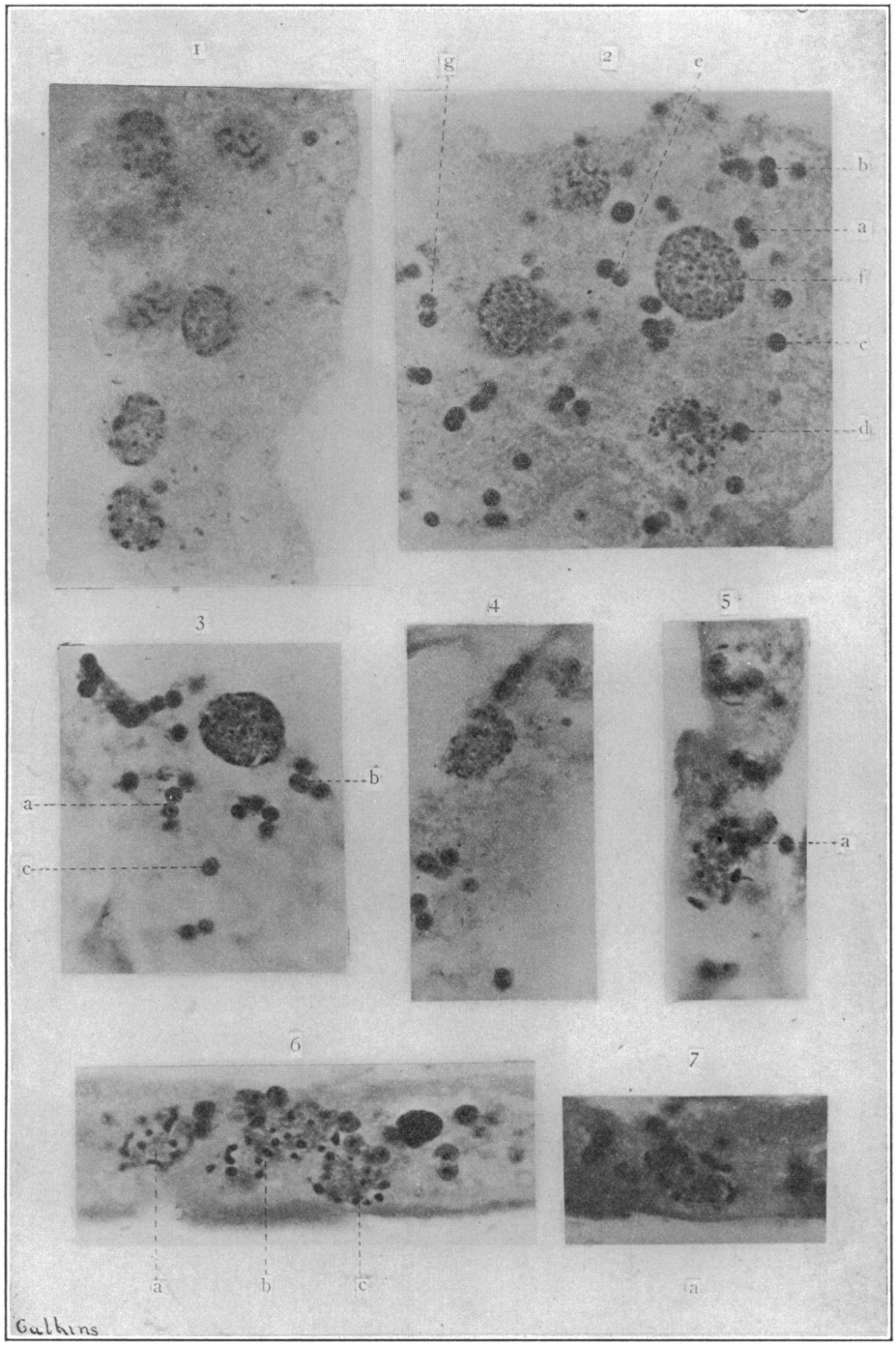
Fig. 4. Primary nucleus with most of the secondary nuclei gone. Some secondary nuclei below in different stages of early fusion, while above are two later stages of union. × 1,500.

Fig. 5. Primary nucleus in the process of liberating secondary nuclei. Two fully formed secondary nuclei are passing into the cytoplasm at (*a*), while others not yet formed remain in the nucleus. Fig. 7 is a deeper section of the same primary nucleus showing the fully formed secondary nuclei within it. × 1,500.

Fig. 6. Section of an amœba in which all the primary nuclei (*a*), (*b*), (*c*) are fragmenting and disintegrating while the secondary nuclei are in various stages of fusion. The section was injured by the objective so that some of the pairs of secondary nuclei are spread. × 1,500.

Fig. 7. Section of same nucleus as that shown in Fig. 5, showing brood (*a*) of secondary nuclei not yet liberated. × 1,500.

[1] The actual dimensions in the photographs must be increased by one fifth to give the magnifications stated.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Explanation of Plate XII.

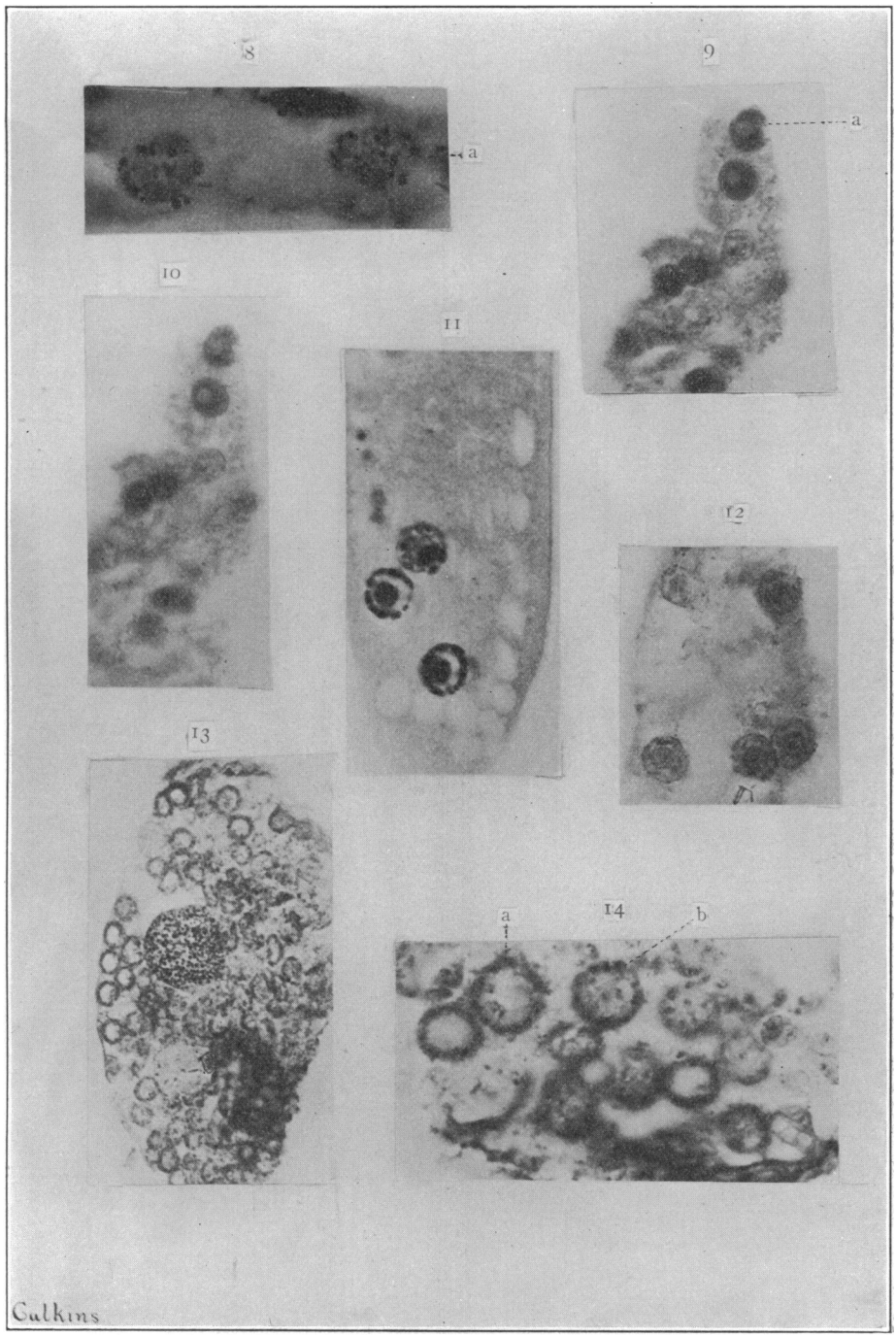
Fig. 8. Two primary nuclei showing broods of secondary nuclei (*a*).

Figs. 9 and 10. Development of the fertilized nuclei, two photographs of the same section at slightly different foci. The upper nucleus at (*a*) shows the characteristic vacuole which becomes the vacuole of the later stages (cf. Figs. 13 and 14). The karyosomes fragment into minute chromatin granules which can be seen in Fig. 9. × 2,000.

Fig. 11. Later stages in development of the fertilized nuclei; the chromatin granules from the disintegrated karyosome now form accumulations about the periphery, these, later, form the nuclei of the spores. × 1,400.

Fig. 12. Intermediate stages in development between that shown in Fig. 9 and that of Fig. 11.

Figs. 13 and 14. Sections of the amœba pictured in Fig. 12 of my earlier paper in the stage of encystment. In Fig. 13 (× 550) the primary nucleus shown is the residual nucleus comparable to the primary residual nucleus in the case of *Polystomella*. Here it is surrounded by many sporoblasts, which, in Fig. 14, are shown more highly magnified (× 1,500). At (*a*) and (*b*) the small but perfect peripheral nuclei may be clearly seen.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